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Proteomic Analyses of a Robust versus a Poor Chicken Gastrointestinal Colonizing Isolate of *Campylobacter jejuni*

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Campylobacter spp. are a significant contributor to the bacterial etiology of acute gastroenteritis in humans. Epidemiological evidence implicates poultry as a major source of the organism for human illness. However, the factors involved in colonization of poultry with *Campylobacter* spp. remain unclear. Determining colonization-associated factors at the proteome level should facilitate our understanding of *Campylobacter* spp. contamination of poultry. Therefore, proteomic analyses were utilized to identify expression differences between two *Campylobacter jejuni* isolates, a robust colonizer A74/C and a poor colonizing strain of the chicken gastrointestinal system designated NCTC 11168-PMSRU. Proteomic analyses by two-dimensional gel electrophoresis revealed the specific expression of an outer membrane-fibronectin binding protein, serine protease, and a putative aminopeptidase in the soluble portion of the robust colonizer A74C. Several proteins including a cysteine synthase and aconitate hydratase were detected specifically in the poor colonizer *C. jejuni* NCTC 11168-PMSRU isolate. Variation in the amino acid sequences resulting in different isoelectric points and relative mobility of the flagellin and *C. jejuni* major outer membrane (MOMP) protein were also detected between the two isolates. Western blotting of the bacterial proteins revealed the presence of two flagellin proteins in the poor colonizer versus one in the robust colonizing isolate, but no differences in MOMP. The results demonstrated that proteomics is useful for characterizing phenotypic variation among *Campylobacter* spp. isolates. Interestingly, different gene products potentially involved in robust colonization of chickens by *Campylobacter* spp. appear to conform to recently identified expression patterns in Biofilm or agar-adapted isolates.

Keywords: epsilon-proteobacteria • zoonotic disease agent • food safety • commensal intestinal bacteria of chickens • phenotypic variation • colonization factors

Introduction

Campylobacter spp. are Gram-negative, microaerophilic bacteria. A majority of *Campylobacter* spp. infections are confined to local acute gastroenteritis characterized by nausea, abdominal cramps, diarrhea, and fatigue caused primarily by *C. jejuni*.[1] Infections are generally self-limited and resolve within several days after initial onset. However, *Campylobacter* spp. infections have also been associated with unnecessary appendectomies, reactive arthritis, and the development of Guillain-Barré syndrome (GBS), a demyelinating neuropathy with acute neuromuscular paralysis. Up to 40% of all human GBS cases in the U.S. occur subsequent to *Campylobacter* spp. infection.[2] While pathogenic in a variety of hosts, *Campylobacter* spp. exist in an apparently commensal relationship with poultry. In the U.S., retail chickens have contamination rates up to 60%–80% with counts approaching 10^6 for fresh and 10^4 for frozen chicken carcasses.[3] The high colonization incidences of poultry by campylobacters and the resultant clinical infections in humans have prompted a number of investigations focused upon identifying and subsequently eliminating *Campylobacter* spp. from poultry. However, the pathways involved in the colonization of poultry flocks remain unclear and multifaceted.[4] Several suspected sources or vectors of contamination include environment of the poultry house, hatchery pads, litter, feed, water, personnel, and small animals on the farm, flies, and rodents.[5–8]

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Although it has been known that many *C. jejuni* isolates may vary in their ability to colonize chicken ceca, nearly all broiler chickens by the age of 30–45 days are colonized with *Campylobacter* spp., and the level of colonization is on average 10^6–10^7 cfu/g of feces.[9] This high level of *Campylobacter* spp. colonization in broilers has been observed throughout the U.S.A.[10,11] It was determined that ciprofloxacin-resistant *Campylobacter* spp. emerged since the 1990s which coincided with the Food and Drug Administration (FDA) approving use of fluoroquinolones in poultry[12] and that 10% of the chicken products purchased from grocery stores contained resistant isolates with 2% of all isolates resistant to erythromycin.[13] Dramatic increases in isolation of fluoroquinolone resistant *C. jejuni* have been reported in the U.S.,[14] and treatment of chickens with fluoroquinolones can induce rapid selection of ciprofloxacin-resistant campylobacters.[15] Class I integron genes associated with potential tobramycin-gentamicin resistance have been detected among *Campylobacter* spp. isolated from U.S. broiler chicken operations.[16] Because of the development of antibiotic resistance among food-borne bacterial agents found in poultry, such as *C. jejuni*, the FDA has discontinued use of enrofloxacin (Baytril) that is utilized for the treatment of colibacillosis in chickens and turkeys.[17]

Currently, the genomic sequences of five *Campylobacter* spp. isolates are publicly available, two *C. jejuni* isolates (11168 and RM1221), one *Campylobacter coli* (RM1228) isolate, one *Campylobacter lari* (RM2100) isolate, and one *Campylobacter upsaliensis* (RM3195) isolate.[18–20] Comparative analysis of the genomes revealed extensive genetic variability among the isolates, but failed to identify specific factors involved with colonization of chickens[19,21] or specific mechanisms associated with bacterial–host interactions.[22] To date, only a limited number of colonization factors have been identified. These include flagellin genes, *sod*, *racR*, *pglH*, and *cadF*.[23–27] Additionally, the importance of iron uptake and metabolism in *C. jejuni* has been reported.[28,29] The role of the ferric uptake regulator (Fur) accompanied by mutations in genes associated with iron metabolism clearly affect the ability of these bacteria to colonize chicken gastrointestinal systems.[30–32] The further identification of genes and gene products involved in *Campylobacter* spp. colonization of chickens is necessary for the development of intervention strategies aimed at the reduction or elimination of *Campylobacter* spp. in poultry. One approach to meet the goal of identifying potential phenotypic differences among these agents is to compare the proteomes of individual isolates that exhibit different phenotypic characteristics. Herein, we report the proteomic analyses between two *C. jejuni* isolates, one a robust and a second poor colonizer of the chicken gastrointestinal system.

Experimental Section

Propagation of *C. jejuni* Isolates and Electron Microscopy. Isolates were propagated in Brucella FBP agar and incubated at 42 °C for 36–48 h in microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) as described previously.[33] The *C. jejuni* isolate NCTC11168 has had the full genome sequenced,[18] and a 11168-PMSRU isolate at our facility has been characterized as a poor colonizer of the chicken gastrointestinal system growing to titers of 10^4 cfu/mL of cecal fluid, while the *C. jejuni* A74C isolate was characterized at our facility as a robust colonizer of the chicken gastrointestinal system growing to titers of 10^7 cfu/mL of cecal fluid in broiler chickens.[34] Electron microscopy was completed by first centrifuging bacteria at 4 °C at 500*g* for 10 min followed by washing in phosphate-buffered saline (PBS; 0.1 M PO_4, 0.8% NaCl, and 0.2% KCl at pH 7) three times. Cells were suspended in 500 μL of 1% gluteraldehyde in PBS, and a 40 μL drop of each sample was placed on parafilm in a Petri dish. A Formvar, carbon-coated 400-mesh grid was placed on each drop of sample and allowed to incubate for 30 min. The grid was placed on a drop of 2% aqueous phosphotungstic acid, pH 7.0, for 30 s. The samples were allowed to dry in a Petri dish lined with filter paper.[35] Once dry, the grids were viewed with a JEM-1210 Transmission Electron Microscope (JEOL USA Inc., Japan) at an acceleration voltage of 120 KeV. Several digital images, as well as corresponding TEM negatives, were taken from random areas on each grid.

Sample Preparation for Two Dimensional (2D) Gel Electrophoresis. Following propagation of the *C. jejuni* isolates in 100 mL of liquid culture, bacteria were centrifuged at 4 °C at 500*g* for 10 min followed by washing in PBS three times. The bacterial pellet was suspended in 40 mM Tris-buffer (pH 8) that contained the protease inhibitor cocktail of aprotinin, bestatin, E-64, leupeptin, and pepstatin (Sigma) followed by sonication. This mixture was centrifuged (500*g*) at 4 °C for 5 min to remove cellular debris. The suspended material was then centrifuged at 16 000*g* at 4 °C for 10 min. The pellet was designated the bacterial membrane protein fraction, while the supernatant was transferred to a separate tube designated the soluble protein fraction. Purification of soluble proteins was completed by adding 4 vol. of cold (−20 °C) acetone for at least 1 h. The soluble protein fraction in acetone was centrifuged at 16 000*g* for 10 min at 4 °C. The pellet was washed in cold acetone/water (4:1) three times followed by centrifugation and dried under vacuum.[36–38] The membrane sample pellet was treated in a similar fashion.

To ensure reproducible gel electrophoresis, both the membrane and soluble protein fractions were digested with *N*-glycosidase F (PNGase F) to cleave oligosaccharides from N-linked glycoproteins[39,40] prior to extraction with acetone as per the manufacturer's instructions (New England BioLab) for 1 h at 37 °C. Additionally, the water/acetone pellets obtained following initial extraction were further purified following the procedure for "2D-gel clean-up" to remove interfering substances such as salts, detergents, lipids or phenolics (Amersham).

Two-Dimensional (2D) Gel Electrophoresis of Bacterial Proteins. Protein samples were suspended in electrophoresis rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3–10, and 0.2% 3/10 ampholyte with 40 mM Tris). Samples were submitted to vortex followed by centrifugation at 16 000*g* at 22 °C for 10 min. Proteomics analyses were completed by 2D gel electrophoresis[41,42] as applied to bacteria.[36–38] Proteins separated as membrane and soluble fractions were suspended in rehydration buffer followed by isoelectric focusing (pH 3–10) in the first dimension, then by SDS-PAGE.[41–43] Spot detection and pattern matching was completed qualitatively by analyzing gels from each isolate in triplicate utilizing PDQuest version 7.3.0 (Bio-Rad, Hercules, CA). Protein spots of interest were cored from 2D-gels and proteins extracted for peptide mass spectrometry fingerprinting. Specifically, spots were cut using BioRad EXQuest spot cutter and digested using Genomic Solutions Proprep.[44]

Identification of Differentially Expressed *C. jejuni* Proteins by Mass Spectrometry. MALDI TOF/TOF analysis[45,46] was used to identify proteins following trypsin digestion. Specifically,

samples were reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide followed by incubation with 75 ng of sequencing grade porcine trypsin (Promega) in 25 mM ammonium bicarbonate for 6 h at 37 °C. Samples were prepared and spotted onto a MALDI (matrix-assisted laser desorption–ionization) target using ZipTipμ-C18 from Millipore. Samples were aspirated and dispensed with ZipTipμ-C18 and eluted with conditioning solution (70% ACN, 0.2% formic acid) containing 5 mg/mL MALDI matrix (α-cyano-4-hydroxycinnamic acid); 0.5 μL was spotted onto the MALDI target.

Samples were analyzed using Applied Biosystems 4700 MALDI TOF/TOF Proteomics Analyzer with TOF/TOF Optics using Applied Biosystems GPS Explorer Software. MALDI-MS data was collected in reflective positive mode (1-keV accelerating voltage) with the m/z range of 800–4000 Da, and 1250 laser shots were averaged for each mass spectrum using m/z of 842.51 and 2211.10 trypsin autolysis products as internal standards. Each sample was internally calibrated if both the 842.51 and 2211.10 ions from trypsin autolysis were present to within 20 ppm. Raw spectrum filtering peak detection settings were S/N threshold of 3, cluster area S/N optimization enabled at S/N threshold 10, baseline subtraction enabled at peak 50. The eight most intense ions from the MS analysis that were not on the exclusion list were subjected to MS/MS. The MS/MS exclusion list included known trypsin masses and unidentified background contamination masses 806.08, 842.52, 843.51, 844.51, 856.52, 857.52, 870.54, 871.54, 872.54, 1011.65, 1045.56, 1126.56, 1338.83, 1420.72, 1531.84, 1666.01, 1794.90, 1940.94, 2003.07, 2211.10, 2225.12, 2239.14, 2283.18, 2299.18, 2678.38, 2807.31, 2914.51, 3094.62, 3337.76, and 3353.75. For the MS/MS analysis, the mass range was 60 Da to precursor ions with a relative precursor window resolution of 50 fwhm (full width at half-maximum). Other settings included an average of 1250 laser shots were collected for each spectrum, CID on, metastable suppressor on. Raw spectrum filtering/peak detection settings were S/N threshold of 5 and cluster area S/N optimization enabled at S/N threshold of 6. The data was stored in an Oracle database (Oracle database schema v. 3.19.0 Data version 3.90.0).

Data was extracted from the Oracle database and peak lists created by GPS Explorer software v. 3.6 (Applied Biosystems). Analyses were performed as combination MS + MS/MS. MS peak filtering included mass range 900–4000 Da, minimum S/N filter 10, and mass exclusion tolerance of 0.2 Da; exclusion list of known trypsin fragments and unidentified background peaks: 2211.1, 2283.2, 1045.6, 842.5, and 1794.9; a peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 65. MSMS peak filtering included mass range of 60–20 Da below each precursor mass. Minimum S/N filter 10, peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 65. The filtered data was searched by Mascot v 1.9.05 (Matrix Science) using Celera Discovery System combined database (CDScombined_KBMS3.0.20041021) containing 1 416 555 sequences. Searches were performed without restriction to protein species, M_r, or pI and with variable oxidation of methionine residues and carbamidomethylation of cysteines. Maximum missed cleavage was set to 1, and searches were limited to trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 20 ppm and ±0.2 Da, respectively.

Peptide Antigens for Antisera Production against *C. jejuni* Major Outer Membrane Protein and Flagellin A Protein. To detect the *C. jejuni* major outer membrane and flagellin proteins, peptide antigens were synthesized to produce affinity-purified antibodies to the proteins. Alignments[47] were conducted with DNA Star to analyze the *C. jejuni* major outer membrane protein (MOMP) and flagellin A (FlaA) for potentially conserved regions predicted to be antigenic[48] amino acid sequences with high surface probability.[49] The peptide sequence N-YRYDTGNFDKNF-C representing residues 40–51 designated MOMP1, N-TGSRLNGDTGRN-C representing residues 325–336 designated MOMP2,[50,51] and sequence N-ATQAAQDGQSLK-C representing residues 96–107 of FlaA[52,53] were utilized as antigens. The peptides were coupled with KLH[54] and utilized to immunize rabbits[55] for antibody production.[56] Subsequently, antibodies were purified by affinity column purification of the rabbit sera[57] as reported by the manufacturer (Sigma-Genosys).

Vertical Gel Electrophoresis by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting. Bacterial isolates were propagated and harvested for SDS-PAGE[58] or 2D-gel electrophoresis as described above and transferred to nitrocellulose[59] to detect reactive proteins. Nonspecific activities were blocked with 1× PBS (1.0 mM sodium phosphate and 15.0 mM sodium chloride) that contained 5% nonfat dry milk (NFDM) with 0.1% Tween 20. Blots were reacted with the primary antibody (diluted 1:1000) for 1 h in blocking solution followed by three washes with 1× PBS and 0.1% Tween 20 (PBST). The blots were then reacted with horseradish-peroxidase-conjugated goat anti-rabbit IgG followed by three washes with PBST. Blots were developed[60] using the Opti-4CN Substrate kit (Bio-Rad). Proteins separated by 2D-gel electrophoresis were treated in the same manner for antibody detection of proteins.

Nucleotide and Predicted Amino Acid Sequence Analyses of the Flagellin, Major Outer Membrane Protein Genes. The flagellin genes (*fla*A and *fla*B) of *C. jejuni* that occur in tandem[61–63] were PCR-amplified from genomic DNA utilizing primers at the 5′ and 3′ ends of the *fla*A gene (FLA4F; FLA1728R) and by utilizing an intervening sequence primer to amplify the *fla*B gene.[64] Additionally, a primer was designed to amplify from the 3′ end of *fla*B gene with the 5′ *fla*A primer FLA4F. The *cmp* gene encoding the major outer membrane protein (MOMP) was amplified utilizing F3 and R3 primers as reported by Zhang et al.[51] All amplification reactions were conducted utilizing Pfu polymerase[65] according to the manufacturer's instructions (Elongase, Invitrogen, Carlsbad, CA) with annealing temperatures as reported by the authors for the primer pairs for each gene with no more than 25 cycles. Oligonucleotide primer sequences utilized for amplification of specific genes are reported in Table 2 of the Supporting Information.

Amplification products were cloned into TA vectors[66] according to the manufacturer's instructions (Invitrogen). DNA from purified plasmids or, in some cases, PCR products directly were sequenced[67] with universal sequencing primers or *C. jejuni*-specific oligonucleotide primers, respectively, using an automated nucleic acid sequencer (Applied Biosystems). Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments[47] were conducted using MacVector 7.2 (Accelrys, San Diego, CA) and DNASTAR (Madison, WI) software. The predicted protein amino acid sequences were

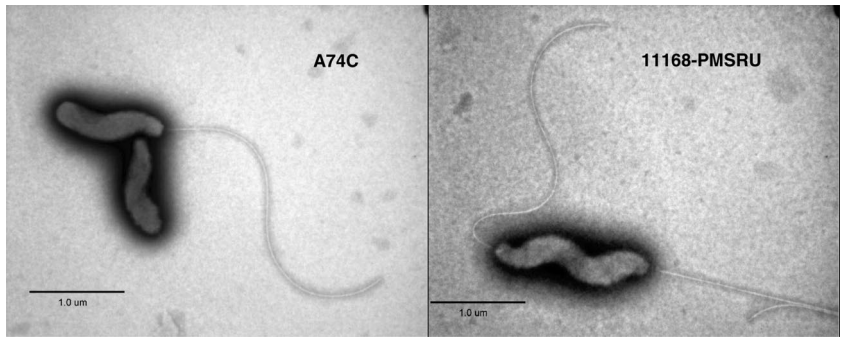

Figure 1. Electron microscopic analyses of poor and robust *C. jejuni* isolates for the chicken gastrointestinal system. Colonies from *C. jejuni* isolates representing a robust chicken colonizer A74C and poor colonizing isolate 11168-PMSRU were visualized by electron microscopy as described in the Experimental Section.

Table 1. Proteins Identified Predominately Expressed by *C. jejuni* Isolate A74C As Determined by Comparative Proteomics

spot no., protein (NCBI accession no.)[a]	ORF[b]	gene[c]	score[d]	cover[e]	peptide[f]
1. Peptidyl-prolyl *cis–trans* isomerase (Q9PJ12)	Cj0115	*slyD*	157	49	7
2. UDP-*N*-acetylglucosamine acyltransferase (Q9PIM1)	Cj0274	*lpxA*	160	31	7
3. Phosphoserine aminotransferase (Q9PIH3)	Cj0326	*serC*	287	40	15
4. Alkyl hydroperoxide reductase subunit C (Q9ZI13)	Cj0334	*ahpC*	199	52	9
5. Putative oxidoreductase subunit (Q9PI91)	Cj0414	–	234	61	11
6. 3-oxoacyl-[acyl-carrier protein] reductase (Q9PI70)	Cj0435	*fabG*	288	50	10
7. 3-oxoacyl-[acyl-carrier-protein] synthase (Q9PI63)	Cj0442	*fabF*	406	60	18
8. Putative secreted protease (Q9PI00)	Cj0511	–	146	34	15
9. Succinyl-CoA synthetase beta chain (Q9PHY1)	Cj0533	*sucC*	218	34	12
10. Inorganic pyrophosphatase (Q9PHM9)	Cj0638c	*ppa*	183	39	8
11. Putative Amino Peptidase P (Q9PHL4)	Cj0653c	–	469	30	16
12. Protein GrpE-HSP-70 cofactor (O69297)	Cj0758	*grpE*	129	40	7
13. Aspartate aminotransferase (Q9PPF7)	Cj0762c	*aspB*	273	28	11
14. Dihydrodipicolinate synthase (Q9PPB4)	Cj0806	*dapA*	704	59	15
15. Ribose-phosphate pyrophosphokinase (Q9PP15)	Cj0918	*prsA*	98	40	10
16. Thiazole biosynthesis protein (Q9PNP6)	Cj1045c	*thiG*	512	50	15
17. Putative glycolate oxidase subunit D (Q9PN82)	Cj1213c	*glcD*	379	44	21
18. Serine Protease (Q9PN69)	Cj1228c	*htrA*	84	28	10
19. Deoxycytidine triphosphate deaminase (Q9PN07)	Cj1292	*dcd*	299	52	7
20. Flavodoxin (Q9PMR8)	Cj1382c	*fldA*	104	20	2
21. Outer Membrane-Fibronectin Binding (Q9X4B1)	Cj1478c	*cadF*	372	47	10

[a] Spot number conforms to the protein designated in Figure 1 with protein name and GenBank accession number (in parenthesis). [b] ORF designation as per *C. jejuni* full genome sequence.[18] [c] Gene or protein designation as standard bacterial identification; dashed line indicates no previous gene designation. [d] Protein Mascot score. [e] Percent sequence coverage. [f] Number of peptides identified.

searched against the protein database by using BLAST[68] and PSI-BLAST or BLASTP,[69,70] as well as the conserved domain database[71] algorithms. Nucleotide sequences for the genes encoding FlaA-FlaB and MOMP proteins of the *C. jejuni* isolate A74C have been submitted to GeneBank with accession number EF660527 for the *flaA* and B genes, while the MOMP gene was assigned accession number EF672033.

Results and Discussion

Two *C. jejuni* isolates previously characterized as poor versus robust colonizers of broiler chickens were subjected to 2D-gel electrophoresis to detect differences in the proteome of each isolate. The *C. jejuni* isolate A74C was determined to colonize chickens at 10^7 cfu/mL, while isolate 11168-PMSRU colonized chicken intestinal systems at 10^4 cfu/mL of cecal fluid. These results indicated that there was a clear distinction in colonizing potential between the two bacterial isolates. Electron microscopy revealed that the robust chicken colonizing isolate (A74C) had a predominance of bacterial morphologies wherein flagella were detected only at one end of the bacterium, whereas the poor colonizing isolate (11168-PMSRU) had primarily two flagella with one at each end of the bacterium (Figure 1) following visualization of one-hundred bacteria per isolate.

Utilizing standard 2D-gel comparisons, we detected several potentially important proteins specifically in the A74C robust colonizing isolate of *C. jejuni* (Table 1 and Figure 2). These included a putative serine protease, putative amino peptidase P, and a branched outer membrane-fibronectin binding protein that have been implicated as potential virulence factors of the bacteria.[18,19] Several other proteins involved with bacterial metabolism[18,19] were also identified by this analysis (Table 1). Alternatively, proteins involved with bacterial metabolism were detected differentially specific in the poor colonizing isolate of *C. jejuni* 11168-PMSRU (Table 2 and Figure 2). These included proteins such as a cysteine synthase, ketol-acid reductoisomerase, and an aconitate hydratase (Figure 2).

The outer membrane-fibronectin binding protein CadF (Cj1478c) detected in the robust colonizing *C. jejuni* isolate A74C (Table 1 and Figure 2) correlates well with previously published information on binding of the bacteria to eukaryotic cells and colonization of chicken gastrointestinal systems. Fibronectin (Fn) is an eukaryotic extracellular adhesion glyco-

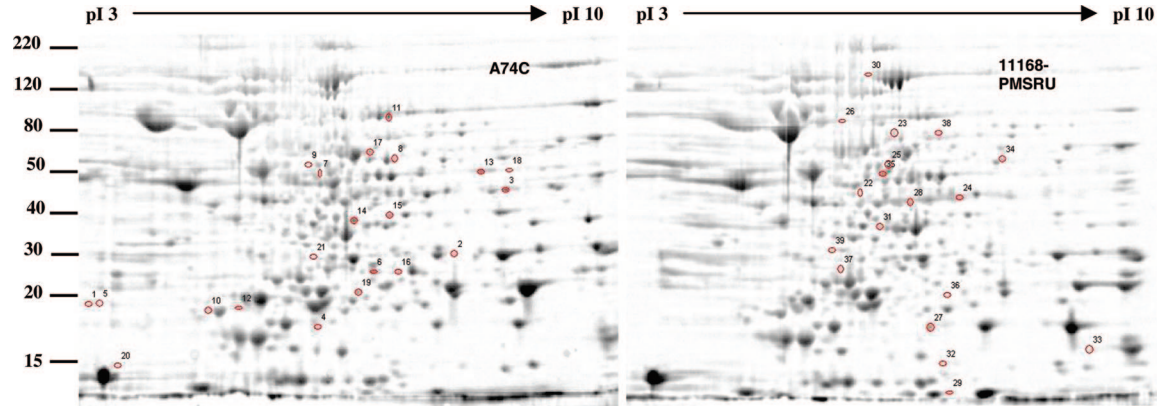

Figure 2. Two-dimensional gel electrophoresis of the soluble portion of the *C. jejuni* proteins. Total proteins were extracted from *C. jejuni* isolates representing a robust chicken colonizer A74C and poor colonizing isolate 11168-PMSRU. Proteins were separated by isoelectric focusing in the first dimension, p*I* 3–10, followed by vertical SDS-PAGE (12.5%) in the second dimension. Spot numbers correspond to proteins listed in Tables 1 and 2.

Table 2. Proteins Identified Predominately Expressed by *C. jejuni* Isolate NCTC 11168-PMSRU As Determined by Comparative Proteomics

spot no., protein (NCBI accession no.)[a]	ORF[b]	gene[c]	score[d]	cover[e]	peptide[f]
22. DNA polymerase III, beta chain (Q9PJA9)	Cj0002	*dnaN*	634	75	27
23. ATP synthase F1 sector alpha subunit (Q9PJ21)	Cj0105	*atpA*	81	20	9
24. Putative cytochrome C551 peroxidase (Q9PIE3)	Cj0358	MauG	180	37	12
25. Serine hydroxymethyltransferase (P24531)	Cj0402	*glyA*	372	57	22
26. Putative GMC oxidoreductase subunit (Q9PI90)	Cj0415	BetA	108	26	13
27. Putative periplasmic protein (Q9PI85)	Cj0420	YceI	200	46	12
28. Ketol-acid Reductoisomerase (Q9PHN5)	Cj0632	*ilvC*	121	45	15
29. Transthyretin-like periplasmic protein (Q9PPJ2)	Cj0715	–	344	47	9
30. Aconitate Hydratase (Q9PP88)	Cj0835c	*acnB*	234	29	19
31. Cysteine Synthase B (P71128)	Cj0912c	*cysM*	127	56	14
32. OMP lipoprotein carrier protein (Q9PNZ0)	Cj0943	*lolA*	102	27	7
33. Ubiquinol-cytochrome *C* reductase subunit (Q9PNA9)	Cj1186c	*petA*	130	44	9
34. Tyrosyl-tRNA synthetase (Q9PN27)	Cj1271c	*tyrS*	276	53	20
35. Malate oxidoreductase (Q9PN12)	Cj1287c	Malic	625	59	20
36. Triosephosphate isomerase (Q9PMQ6)	Cj1401c	*tpiA*	110	32	10
37. FdhD (formate dehydrogenase D) (Q9PMF5)	Cj1508c	*fdhD*	217	67	14
38. Putative peptide ABC-transport peptide (Q9PM91)	Cj1584c	–	93	31	15
39. Curamycin polyketide acyl carrier protein (Q02570)[g]	–	*cure*	55	52	3

[a] Spot number conforms to the protein designated in Figure 2 with protein name and GenBank accession number (in parenthesis). [b] ORF designation as per *C. jejuni* full genome sequence.[18] [c] Gene designation as standard bacterial identification; dashed line indicates no gene designation. [d] Protein Mascot score. [e] Percent sequence coverage. [f] Number of peptides identified. [g] Protein from *C. jejuni* not identified previously in the full-genome sequence; polyketide synthesis-encoding gene cluster member of *Streptomyces cyaneus*.

protein that facilitates adherence of microorganizms including *C. jejuni* mediated by CadF[72] that contains a Fn-binding domain.[73] The *cadF* gene was reported as being conserved among campylobacters,[74] and the CadF protein is potentially required for colonization of chickens.[27] Consequently, the presence of this protein in the proteome of the robust colonizing isolate is consistent with previous observations.

The HtrA serine protease (Cj1228c) was specifically expressed in the robust colonizing isolate A74C (Table 1 and Figure 1). The *htrA* gene encoding the stress protein HtrA, a periplasmic protease, is essential for intracellular replication of adherent and invasive *Escherichia coli* within J774-A1 macrophages. Deletion of the *htrA* gene in this bacterium induced increased sensitivity of an isogenic mutant to oxidative stress.[75] Lack of HtrA also reduces the oxygen tolerance of *C. jejuni*, and an *htrA* mutant adhered to and invaded human epithelial INT407 cells with a decreased frequency compared to the wild-type strain.[76] A putative amino peptidase P (APP; Cj0653c) was first described in *E. coli*[77] and has been demonstrated in a number of lactococcal strains to contribute to the abolition of bitterness during the ripening of cheese by participating in peptide degradation following release into the matrix.[78] Consequently, the role this protein may play in *C. jejuni* virulence is unknown, although it is expressed in the robust colonizer A74C (Table 1).

Several other potentially important proteins (Table 1 and Figure 2) including a UDP-*N*-acetylglucosamine acyltransferase (Cj0274) encoding the *lpxA* gene that catalyzes the first step of lipid A biosynthesis and subsequently lipopolysaccharide biosynthesis[79] were detected in the robust colonizer A74C. The alkyl hydroperoxide reductase C (Cj0334) gene was present in the A74C isolate, and this protein is important in the defense against oxidative stress.[80] The 3-oxoacyl-[acyl-carrier protein] reductase (Cj0435) and synthase (Cj0442) genes encode proteins that are characteristically disulfide reductases that provide resistance to drugs and elimination of toxic compounds,[81] while proteins such as the GrpE (encoded by Cj0758) may increase the ability of Campylobacter to adhere to and invade human epithelial INT-407 cells.[82]

The isolate 11168-PMSRU characterized as a poor colonizer of chickens was reported to express an aconitate hydratase (Cj0835c), cysteine synthase (Cj0912c), and ketol-acid reductoisomerase (Cj0632) relative to the robust colonizing isolate A74C (Table 2 and Figure 2). In addition to being the major citric acid cycle iron-sensitive aconitase[83] in *E. coli,* the aconitase B protein (AcnB) is also a post-transcriptional regulator of gene expression.[84] One role for AcnB is initiation of a regulatory process controlling flagella biosynthesis in *Salmonella enterica*.[85] Consequently, increased expression or presence of this enzyme may not only function in the anaplerotic reactions for *C. jejuni* energy production,[86] but could also play a role in regulating expression of flagella biosynthesis.

Cysteine synthase (Cj0912c) or *O*-acetylserine sulfhydrylase B in *E. coli* catalyzes the formation of L-cysteine from *O*-acetylserine, and sulfide has been characterized in C. *jejuni*[87] and was expressed predominantly in the 11168-PMSRU isolate (Table 2 and Figure 2). Mutation of the *cysM* gene significantly reduced the ability of *Staphylococcus aureus* to recover from starvation in amino acid- or phosphate-limiting conditions, indicating a role for cysteine in the *S. aureus* as a potential survival mechanism.[88] Cysteine is preferentially utilized during growth of *E. coli* under anaerobic conditions, and *E. coli* complemented with the *C. jejuni cysM* gene was capable of growth in medium containing either sulfide or thiosulfate as sole sulfur sources.[87] A ketol-acid reductoisomerase (Cj0632; IlvC) that catalyzes two steps in the biosynthesis of branched-chain amino acids important in the metabolism of these intermediates[89] was also expressed predominantly in the poor-colonizing *C. jejuni* isolate (Table 2 and Figure 2).

Interestingly, levels of DnaN (Cj0002) were increased in the 11168-PMSRU isolate (Table 2 and Figure 2). The DnaK and ClpB proteins were increased in an *htrA* mutant, and the *htrA* mutant adhered to and invaded human epithelial cells with a decreased frequency compared to wild-type strain.[76] Also, a curamycin polyketide acyl carrier protein was identified in the 11168-PMSRU isolate (Table 2 and Figure 2) that has not been previously reported in this bacterium. The curamycin polyketide acyl carrier protein was most similar to the protein found in *Streptomyces cyaneus*.[90] Polyketide synthesis is involved with the condensation of acyl-CoA with methylmalonyl-CoA to form methyl-branched fatty acid, a reaction recently shown to be catalyzed in mycobacteria by a family of enzymes called type I polyketide synthases.[91]

Since sequence differences among the MOMP[49,92] and flagellin[25,52,93] proteins may be detected among *C. jejuni* isolates, peptide antibodies were generated against these proteins and were utilized for Western blot detection of the proteins between the two isolates (Figure 3 and Supplemental Figure 1 in Supporting Information). Antibodies to the MOMP only detected one protein for both isolates which did not vary in relative mobility (Supplemental Figure 1 in Supporting Information). The MOMP proteins between 11168-PMSRU and A74C were 99% similar with a T for R difference at position 268 and an A for T difference at residue 368 between 11168-PMSRU and A74C, respectively (Supplemental Figure 2 in Supporting Information). The amino acid residues utilized to generate peptide antibodies for MOMP protein were invariant between the two isolates. Although differences among *C. jejuni* MOMP porin proteins can be detected, this phenomenon[49] does not appear to play a role in pathogenesis if a functional molecule is expressed by the bacterium.[92]

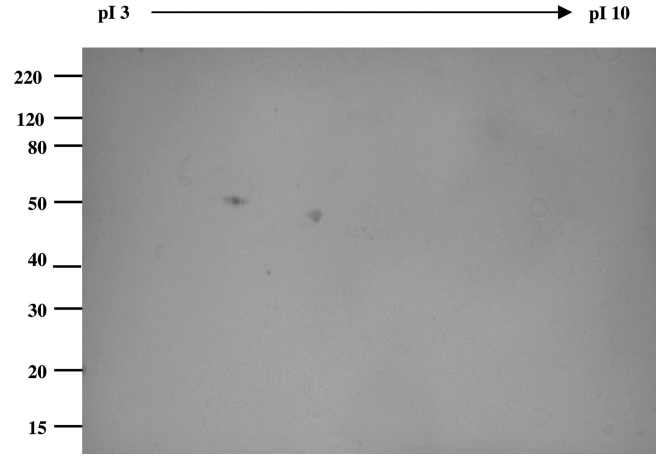

Figure 3. Immunological detection of *C. jejuni* flagellin proteins. Membrane protein fractions were extracted from *C. jejuni* isolate representing the poor chicken colonizer 11168. Proteins were separated by isoelectric focusing in the first dimension, p*I* 3–10, followed by vertical SDS-PAGE (8–12%) in the second dimension and electro-blotting with detection using affinity-purified antibody to the flagellin A protein.

With the use of the flagellin antibody, two proteins that differed in both relative mobility and isoelectric point were detected in the poor colonizing isolate 11168 (Figure 3 and Supplemental Figure 1 in Supporting Information), while only the larger protein was detected in the robust colonizing isolate A74C (Supplemental Figure 1 in Supporting Information). Sequence differences did not occur between the two isolates relative to the peptide antigen utilized to generate antibodies to FlaA or FlaB (Figure 4). The FlaA and FlaB proteins of 11168-PMSRU were 100% similar to the published 11168 sequence (Parkhill et al.[18]), but both were only 81% similar to the A74C isolate's respective proteins. The 11168-PMSRU FlaA and FlaB proteins were most similar (99%) to the proteins from *C. jejuni* isolate CF93–6 (EAQ56833) and 84–25 (EAQ95644) for which there is no biological information (Fouts and Nelson, unpublished) and isolates L17 and D2290.[64] The A74C FlaA and FlaB proteins were 100% similar to its parent strain A74/O[64] and greater than 99% similar to *C. jejuni* isolate 81116[61,63] and isolate 81–176.[94]

The nineteen S and T residues of the flagellin central domain potentially exposed to the external environment that are modified by addition of pseudaminic acid analogues[94] are present in the A74C protein, but not all are present in the FlaA or FlaB of the 11168-PMSRU isolate (Figure 4). Specifically, there were several deletions in the 11168-PMSRU proteins at residues 389–391 and at position 437 relative to numbering of the 81–176 protein.[94] Also, there was an L for T substitution at residue 394, an A for S at position 430, the deletion of the S at position 437, and a Q for S substitution at residue 440 that would account for lack of glycosylation at those residues in the 11168-PMSRU FlaA and Fla B proteins. The 5′ and 3′ regions of the A74C *fla*A and *fla*B genomic regions are conserved relative to the 81116 isolate, whereas the 11168-PMSRU has deleted sequences in the IVS (data not shown). The results confirm conclusions by Fagerquist et al.[95] that biomarker mass shifts between *C. jejuni* strains were due to differences in primary sequence of the protein and not to post-translational modifications.

Biofilm-propagated *C. jejuni* on sterile membranes[96] had up-regulation of genes expressing proteins involved with motility

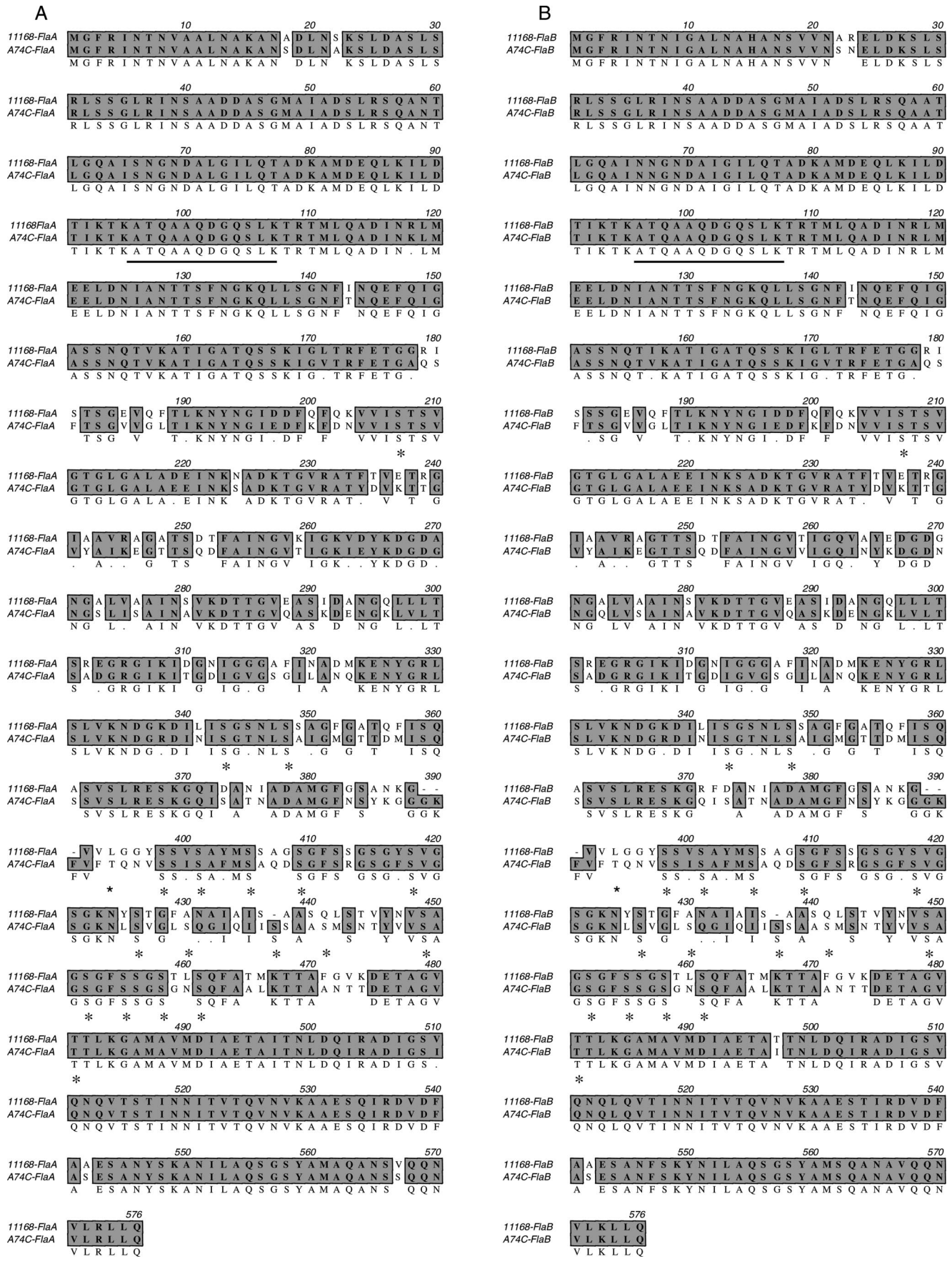

Figure 4. Predicted amino acid sequence alignment of the *C. jejuni* FlaA and FlaB proteins for the 11168-PMSRU and A74C isolates. After translation of the respective gene sequences, amino acid sequences were aligned by Clustal W. Underlined sequences conform to the peptide utilized to generate antisera, while asterisks denote potential glycosylation sites.

such as FlaA and FlaB along with proteins involved with oxidative stress and adhesions such as Peb1 and FlaC. Peb1 is a component of a putative ABC transport system that binds aspartate/glutamate.[97] Although propagation of the robust colonizing isolate A74C was not completed on surfaces, the bacterium did specifically express a fibronectin-binding outer membrane protein CadF along with an HtrA serine protease important for binding and eukaryotic cell invasion, respectively (Table 1). The findings reported herein are homologous to gene expression following propagation of *C. jejuni* on membranes[96] or agar[98] versus planktonic propagation of the bacterium in broth. It was concluded that agar growth overlapped expression of proteins during host colonization. Growth in broth induced expression of proteins involved with oxidative phosphorylation along with nitrogen and energy metabolism.[98] Also, it is known that different isolates have varying abilities to form biofilms that may be related to an isolate's persistence in the environment.[99]

Conclusions

These results demonstrate that comparative proteomics is a useful tool for the identification of genomic variation between C. *jejuni* isolates. Identification of different gene products potentially involved with colonization of *C. jejuni* in poultry will facilitate the development of targeted intervention strategies, such as oral vaccination, for reduction of these bacteria among chickens. The data reported herein may also support the hypothesis that robust colonizing isolates of *C. jejuni* may genetically resemble the expression patterns of isolates in biofilms or that are propagated on agar versus broth culture and that poor colonizing isolates of this bacteria more resemble gene expression by planktonic *C. jejuni* cells in culture.

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Supporting Information Available: Tables listing the proteins identified with their corresponding MS data and the primers used in this study to amplify and sequence *C. jejuni* specific genes; figures of SDS-PAGE and Western blots and MOMP alignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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